

## **A REPRESSOR-MEDIATED REGULATION SYSTEM FOR CONTROL OF GENE EXPRESSION IN PLANTS**

5 The present invention relates to the regulation of gene expression. More particularly, the present invention pertains to the control of gene expression of one or more nucleotide sequences of interest in transgenic plants using a repressor protein and corresponding operator sequences.

### **BACKGROUND OF THE INVENTION**

10

Transgenic plants have been an integral component of advances made in agricultural biotechnology. They are necessary tools for the production of plants exhibiting desirable traits (e.g. herbicide and insect resistance, drought and cold tolerance), or producing products of nutritional or pharmaceutical importance. As the applications of transgenic plants become ever more sophisticated, it is becoming increasingly necessary to develop strategies to fine-tune the expression of introduced genes. The ability to tightly regulate the expression of transgenes is important to address many safety, regulatory and practical issues. To this end, it is necessary to develop tools and strategies to regulate the expression of transgenes in a predictable manner.

20

Several strategies have so far been employed to control plant gene/transgene expression. These include the use of regulated promoters, such as inducible or developmental promoters, whereby the expression of genes of interest is driven by promoters responsive to various regulatory factors (Gatz, 1997, *Ann. Rev. Plant Physiol. Plant Mol. Biol.*, 48: 89). Other strategies involve co-suppression (Eisner et al., 1998, *Ther. Appl. Genet.*, 97: 801) or anti-sense technology (Kohno-Murase et al., 1994, *Plant Mol. Biol.*, 26: 1115), whereby plants are transformed with genes, or fragments thereof, that are homologous to genes either in the sense or antisense orientations. Chimeric RNA-DNA oligonucleotides have also been used to block the expression of target genes in plants (Beetham et al., 1999, *Proc. Natl. Acad. Sci. USA*, 96: 8774; Zhu et al., 1999, *Proc. Natl. Acad. Sci. USA*, 96: 8768).

30

The ROS protein is encoded by the chromosomal gene, ROS, of *Agrobacterium tumefaciens*. In this organism, the ROS protein acts as a negative regulator for the expression of the Ti-plasmid-encoded VirC, VirD and IPT genes (Cooley et al., J. 1991, Bacteriol. 173: 2608-2616; Chou et al., 1998, Proc. Natl. Acad. Sci., 95: 5293; Archdeacon J et al. 2000, FEMS Microbiol Let. 187: 175-178; D'Souza-Ault M. R., 1993, J Bacteriol 175: 3486-3490). The ROS protein is a DNA binding protein that is able to bind a ROS operator sequence ( D'Souza-Ault M. R., 1993, J Bacteriol 175: 3486-3490).

Analysis of the amino acid sequence of the ROS protein reveals that it has a DNA binding motif of the C<sub>2</sub>H<sub>2</sub> zinc finger configuration (Chou et al., 1998, Proc. Natl. Acad. Sci., 95: 5293). Typical zinc fingers are characterised by the presence of two cysteine and two histidine residues joined together by the coordination of a single zinc ion. A stretch of amino acids forms a peptide loop, known as the zinc finger motif that is required for DNA binding. Zinc finger proteins represent a significant portion of proteins in eukaryotes, but are rare in prokaryotes. The zinc finger of the bacterial ROS protein varies from its counterparts in eukaryotes in that the ROS protein has only one zinc finger motif, while eukaryotic zinc finger proteins have multiple zinc finger motifs. In addition, there are 9 amino acid residues making up the peptide loop spacing the zinc finger motif in the ROS protein as compared to the 12 amino acids that make up the loops of zinc fingers of eukaryotic proteins.

There is no suggestion for the use of ROS repressor to regulate gene expression within plants. The present invention provides a method for the regulation of gene expression in plants using a nucleic acid sequence, or derivatives of thereof, that encode ROS.

It is an object of the invention to overcome disadvantages of the prior art.

The above object is met by the combinations of features of the main claims, the sub-claims disclose further advantageous embodiments of the invention.

5     **SUMMARY OF THE INVENTION**

          The present invention relates to the regulation of gene expression. More particularly, the present invention pertains to the control of gene expression of one or more nucleotide sequences of interest in transgenic plants using a repressor protein and  
10    corresponding operator sequences.

          According to the present invention there is provided a method (A) for selectively controlling the transcription of a gene of interest, comprising:

- 15    i)   producing a first plant comprising a first genetic construct, the first genetic construct comprising a first regulatory region operatively linked to a gene of interest and at least one repressor operator sequence capable of controlling the activity of the first regulatory region;
- 20    ii)   producing a second plant comprising a second genetic construct, the second genetic construct comprising a second regulatory region in operative association with a nucleic acid molecule, or a derivative thereof, encoding a repressor, the repressor exhibiting both repressor operator binding activity and repressor activity;
- 25    iii)   crossing the first plant and the second plant to obtain progeny, the progeny comprising both the first genetic construct and the second genetic construct, and characterized in that the expression of the second genetic construct represses expression of the gene of interest.

          It is preferred that the gene encoding the repressor is optimized for expression in the plant, and that the gene encodes a nuclear localization signal. Furthermore, it is preferred that the repressor is a ROS repressor, and the repressor operator sequence is a ROS  
30    operator sequence.

The present invention also embraces the above method (A), wherein the first and second regulatory regions are either the same or different and are selected from the group consisting of a constitutive promoter, an inducible promoter, a tissue specific promoter, and a developmental promoter.

5

The present invention further provides a method (B) for selectively controlling the transcription of a gene of interest in a plant, comprising:

- i) introducing into the plant either separately, or within the same vector:
  - 10 a) a first genetic construct comprising a nucleic acid molecule comprising a first regulatory region operatively linked to a gene of interest, and at least one ROS operator sequence capable of controlling the activity of the first regulatory region; and
  - b) a second genetic construct comprising a second regulatory region in operative association with a nucleotide sequence encoding a ROS repressor, or a derivative thereof, said ROS repressor exhibiting ROS operator binding activity, ROS repressor activity or both ROS operator binding activity and ROS repressor activity, the second regulatory region  
15 comprises an inducible promoter;
- ii) growing the plant, and
- 20 iii) inducing the activity of said inducible promoter so that expression of the second genetic construct produces the ROS repressor and represses expression of the gene of interest.

It is preferred that the gene encoding the repressor is optimized for expression in the plant, and that the gene encodes a nuclear localization signal. Furthermore, it is preferred  
25 that the repressor is a ROS repressor, and the repressor operator sequence is a ROS operator sequence.

The present invention embraces a method (C) for selectively controlling the transcription of a gene of interest in a plant, comprising:

- 30 i) introducing into the plant either separately, or within the same vector::

- a) a first genetic construct comprising a nucleic acid molecule comprising a first regulatory region operatively linked to a gene of interest, and at least one ROS operator sequence capable of controlling the activity of the first regulatory region; and
- 5 b) a second genetic construct comprising a second regulatory region in operative association with a nucleotide sequence encoding a ROS repressor, or a derivative thereof, said ROS repressor exhibiting ROS operator binding activity, ROS repressor activity, or both ROS operator binding activity ROS repressor activity; the second regulatory region comprises a tissue specific promoter; and
- 10 ii) growing said plant, so that expression of said second genetic construct produces said ROS repressor and represses expression of said gene of interest in a tissue specific manner.

15 It is preferred that the gene encoding the repressor is optimized for expression in the plant, and that the gene encodes a nuclear localization signal. Furthermore, it is preferred that the repressor is a ROS repressor, and the repressor operator sequence is a ROS operator sequence.

20 The present invention also provides a method (D) for selectively controlling the transcription of a gene of interest in a plant, comprising:

- i) introducing into the plant either separately, or within the same vector:
  - a) a first genetic construct comprising a nucleic acid molecule comprising a first regulatory region operatively linked to a gene of interest, and at least one ROS operator sequence capable of controlling the activity of the first regulatory region; and
  - 25 b) a second genetic construct comprising a second regulatory region in operative association with a nucleotide sequence encoding a ROS repressor, or a derivative thereof, said ROS repressor exhibiting ROS operator binding activity, ROS repressor activity, or both ROS operator binding activity ROS repressor activity; second regulatory region
- 30

comprises a promoter that is active at one or more specific developmental stages within the plant; and

- ii) growing the plant, so that the activity of the promoter at one or more specific developmental stages within the plant results in expression of the second genetic construct thereby producing said ROS repressor, and represses expression of the gene of interest.

It is preferred that the gene encoding the repressor is optimized for expression in the plant, and that the gene encodes a nuclear localization signal. Furthermore, it is preferred that the repressor is a ROS repressor, and the repressor operator sequence is a ROS operator sequence.

The present invention is also directed to a nucleic acid molecule, or a derivative thereof, encoding a ROS repressor optimized for plant codon usage and exhibiting both ROS operator binding activity and ROS repressor activity. The nucleic acid molecule or a derivative thereof, may be characterized as comprising one or more of the following properties:

- a) comprising greater than 80% similarity with the nucleotide sequence of SEQ ID NO:2 or 3 as determined by use of the BLAST algorithm with the following parameters: blastn; Database: nr; Expect 10; filter: low complexity; Alignment: pairwise; Word Size:11;
- b) hybridizing under stringent conditions with the nucleotide sequence of SEQ ID NO:2 or 3, comprising hybridizing for 16-20 hrs at 65°C in 7% SDS, 1mM EDTA, 0.5M Na<sub>2</sub>HPO<sub>4</sub>, pH 7.2, followed by washing in 5% SDS, 1mM EDTA 40mM Na<sub>2</sub>HPO<sub>4</sub>, pH 7.2 for 30 min, followed by washing in 1% SDS, 1mM EDTA 40mM Na<sub>2</sub>HPO<sub>4</sub>, pH 7.2 for 30 min;
- c) comprising the nucleotide sequence of SEQ ID NO:2; and
- d) comprising the nucleotide sequence of SEQ ID NO:3.

Furthermore, the present invention relates to a genetic construct comprising a regulatory region in operative association with the nucleic acid molecule as defined above, and to a plant, or seed comprising the genetic construct.

The present invention also pertains to a nucleic acid molecule as defined above, further comprising a nuclear localization signal fused to the nucleic acid molecule, and to a genetic construct comprising a nuclear localization signal fused to the nucleic acid molecule as defined above. The present invention includes, a plant, or seed comprising  
5 the genetic construct as just defined.

The present invention further relates to a nucleic acid molecule comprising a regulatory region operatively linked to a gene of interest and at least one ROS operator sequence capable of controlling the activity of the regulatory region, wherein the  
10 regulatory region is functional in plants. Preferably, the at least one ROS operator sequence comprises the nucleotide sequence of SEQ ID NO:8. This invention also provides a genetic construct comprising the nucleic acid molecule as just defined, and to a plant comprising the genetic construct.

The present invention also pertains to a plant comprising a first genetic construct comprising a first nucleic acid molecule comprising a first regulatory region operatively linked to a gene of interest, and at least one ROS operator sequence capable of controlling the activity of the first regulatory region, and a second genetic construct comprising a second nucleic acid molecule, or a derivative thereof, encoding a ROS  
15 repressor optimized for plant codon usage and exhibiting ROS operator binding activity, ROS repressor activity, or both ROS operator binding activity ROS repressor activity.  
20

This summary of the invention does not necessarily describe all necessary features of the invention but that the invention may also reside in a sub-combination of the  
25 described features.

## BRIEF DESCRIPTION OF THE DRAWINGS

These and other features of the invention will become more apparent from the following description in which reference is made to the appended drawings wherein:

5 **FIGURE 1** shows the nucleotide and deduced amino acid sequences of wild type ROS and a modified ROS of *Agrobacterium tumefaciens*. **Figure 1(A)** shows the amino acid sequence alignment of known ROS repressors, and a synthetic ROS. The amino acid sequence 'PKKKRKV' at the carboxy end of synthetic ROS is one of several nuclear localization signals. **Figure 1(B)** shows the nucleotide  
10 sequence of a synthetic ROS that had been optimized for plant codon usage containing a nuclear localization signal peptide (in italics). Optional restriction sites at the 5' end of the sequence are underlined (also see SEQ ID NO:2). **Figure 1(C)** shows the consensus nucleotide (SEQ ID NO:3) and predicted amino acid sequence, of a composite ROS sequence comprising all possible nucleotide  
15 sequences that encode wild type ROS repressor, and the wild type ROS amino acid sequence. The amino acid sequence 'PKKKRKV' at the carboxy end represents a nuclear localization signal. Amino acids in bold identify the zinc finger motif. Nucleotide codes are as follows: N= A or C or T or G; R= A or G; Y= C or T; M= A or C; K= T or G; S= C or G; W= A or T; H= A or T or C; B= T or C or G; D= A or T or G; V= A or C or G. **Figure 1(D)** shows the  
20 nucleotide sequence of the DNA binding sites (operator sequences) of the virC/virD and ipt genes. **Figure 1(E)** shows a consensus operator sequence derived from the virC/virD and ipt operator sequences (SEQ ID NO:20). This sequence comprises 10 nucleotides, however, only the first 9 nucleotides are  
25 required for binding ROS.

**FIGURE 2** displays the structure of various constructs in which the transcription of a modified ROS or wild type ROS nucleotide sequence is placed under control of various regulatory regions. The modified ROS nucleotide sequence is designated  
30 as 'synthetic ROS'. **Figure 2(A)** shows a schematic diagram of the p74-107 nucleotide construct in which a CaMV35S regulatory region is operatively linked



to the wild type ROS protein coding region. **Figure 2(B)** shows the nucleotide construct p74-313 in which a CaMV35S regulatory region is operatively linked (transcriptionally fused) to the protein coding region of synthetic ROS. **Figure 2(C)** shows the nucleotide construct p74-108 in which a tms2 regulatory region is transcriptionally fused to the protein coding region of synthetic ROS. **Figure 2(D)** shows the nucleotide construct p74-101 in which an actin2 regulatory region is operatively linked to the protein coding region of synthetic ROS.

**FIGURE 3** shows schematic representations of nucleotide constructs that place the expression of a gene of interest under the control a regulatory region, in this case a CaMV35S regulatory region, modified to contain a ROS operator site. **Figure 3(A)** shows the nucleotide construct p74-315 in which a CaMV35S regulatory region, modified to contain a ROS operator site downstream of the TATA box, is operatively linked to a gene of interest ( $\beta$ -glucuronidase; GUS). **Figure 3(B)** shows the nucleotide construct p74-316 in which a CaMV35S regulatory region is modified to contain a ROS operator site upstream of the TATA box is operatively linked to the protein encoding region of GUS. **Figure 3(C)** shows the nucleotide construct p74-309 in which a CaMV35S regulatory region modified to contain ROS operator sites upstream and downstream of the TATA box is transcriptionally fused (i.e. operatively linked) to the protein encoding region of GUS.

**FIGURE 4** shows a schematic representation of a nucleotide construct that places the expression of a gene of interest gene under the control of a regulatory region, in this case, the tms2 regulatory region that has been modified to contain ROS operator sites. **Figure 4(A)** shows the nucleotide construct p76-507 in which a tms2 regulatory region is operatively linked to a gene of interest (in this case encoding  $\beta$ -glucuronidase, GUS). **Figure 4(B)** shows the nucleotide construct p76-508 in which a tms2 regulatory region modified to contain two tandemly repeated ROS operator sites downstream of the TATA box is transcriptionally fused (i.e. operatively linked) to the protein coding region of GUS.

FIGURE 5 shows a schematic representation of a nucleotide construct that places the expression of a gene of interest under the control of a regulatory region, in this case actin 2 regulatory region, that has been modified to contain ROS operator sites. Figure 5(A) shows the nucleotide construct p75-101 in which an actin2 regulatory region is operatively linked to a gene of interest (the  $\beta$ -glucuronidase (GUS) reporter gene). Figure 5(B) shows the nucleotide construct p74-501 in which an actin2 regulatory region modified to contain two tandemly repeated ROS operator sites upstream of the TATA box is transcriptionally fused (operatively linked) to the a gene of interest (GUS). Figure 5C shows construct p74-118 comprising a 35S regulatory region with three ROS operator sites downstream from the TATA box. The 35S regulatory region is operatively linked to the gene of interest (GUS).

FIGURE 6 shows Southern analysis of transgenic *Arabidopsis* plants. Figure 6(A) shows Southern analysis of a plant comprising a first genetic construct, p74-309 (35S-operator sequence-GUS; see Figure 3(C) for map). Figure 6(B) shows Southern analysis of a plant comprising a second genetic construct, p74-101 (actin2-synthetic ROS; see Figure 2(D) for map).

FIGURE 7 shows Westerns analysis of ROS expression in transformed *Arabidopsis* plants. Levels of wild type ROS, p74-107 (35S-WTROS; see Figure 2(A) for map), and synthetic ROS p74-101 (actin2-synROS; see Figure 2(D) for map) produced in transgenic plants were determined by Western analysis using a ROS polyclonal antibody. *Arabidopsis* var. columbia, was run as a control.

FIGURE 8 shows expression of a gene of interest in plants. Upper panel shows expression of GUS under the control of 35S (pBI121; 35S:GUS). Middle panel shows GUS expression under the control of actin2 comprising ROS operator sequences (p74-501; see Figure 5(B) for construct). Lower panel shows the lack of GUS activity in a non-transformed control.

**FIGURE 9** shows regulation of a gene of interest in progeny plants arising from a cross between a ROS parent plant (expressing p74-101, Figure 2D) and a plant expressing a gene of interest under the control of a regulatory region comprising ROS operator sequences (GUS parent expressing p74-118, Figure 5C). **Figure 9A** shows GUS activity in the ROS and GUS parents and the progeny obtained from the cross of the ROS and GUS parents. **Figure 9B** shows Northern analysis of RNA obtained from ROS and GUS parents and the progeny of the cross between the ROS and GUS parents and probed with either a ROS or GUS probe. **Figure 9C** shows Southern analysis of the progeny of the cross between the GUS and ROS parent plants, probed with either a GUS or ROS probe.

## DESCRIPTION OF PREFERRED EMBODIMENT

The present invention relates to the regulation of gene expression. More particularly, the present invention pertains to the control of gene expression of one or more nucleotide sequences of interest in transgenic plants using a repressor protein and corresponding operator sequences.

The following description is of a preferred embodiment by way of example only and without limitation to the combination of features necessary for carrying the invention into effect.

Gene repression can be used in applications such as metabolic engineering to produce plants that accumulate large amounts of certain intermediate compounds. Repression of gene expression can also be used for control of transgenes across generations, or production of F1 hybrid plants with seed characteristics that would be undesirable in the parental line, for example but not limited to, hyper-high oil, reduced fiber content, low glucosinolate levels, reduced levels of phytotoxins, and the like. In the latter examples, low glucosinolate levels, or other phytotoxins, may be desired in seeds while higher concentrations of these compounds may be required elsewhere, for example in the case of glucosinolates, within cotyledons, due to their role in plant defence. Another non-limiting example for the controlled regulation of a gene of interest during plant development is seed specific down regulation of sinapine biosynthesis, as for example in seeds of *Brassica napus*. In many instances, transgene expression needs to be repressed only in certain plant organs/tissues or at certain stages of development. The methods as described herein may also be used to control the expression of a gene of interest that encodes a protein used to for plant selection purposes. For example, which is to be considered non-limiting, a gene of interest may encode a protein that is capable of metabolizing a compound from a non-toxic form to a toxic form thereby selectively removing plants that express the gene of interest.

The present invention is directed to a method of controlling gene expression using a repressor protein as a regulatory switch to repress the expression of a gene of interest or repress the transcription of one or more selected nucleotide sequences by transforming a plant with one or more constructs comprising:

5           1) a first nucleotide sequence comprising a gene of interest operatively linked to a regulatory region comprising at least one repressor operator sequence that interacts with a repressor protein.

          2) a second nucleotide sequence comprising a regulatory region in operative association with a nucleotide sequence encoding the repressor protein.

10       Preferably the repressor protein is ROS, and the repressor operator sequence is a ROS repressor operator sequence, for example but not limited to the ROS repressor encoded by the nucleic acid sequence of SEQ ID NO:3.

          These first and second nucleotide sequences may be placed within the same or within different vectors, genetic constructs, or nucleic acid molecules. When both  
15       constructs are expressed within the same plant, the expression of the repressor protein results in the down regulation in the expression of a gene of interest that is in operative association with an operator sequence that exhibits an affinity for the repressor protein.

20       By the term "expression" it is meant the production of a functional RNA, protein or both, from a gene or transgene.

          By "repression of gene expression" it is meant the reduction in the level of mRNA, protein, or both mRNA and protein, encoded by a gene or nucleotide sequence  
25       of interest. Repression of gene expression may also arise as a result of the lack of production of full length RNA, for example mRNA, due to blocking migration of polymerase along a nucleic acid during transcription. A repression of gene expression may be a consequence of repressing, blocking or interrupting transcription.

30       By "repressor" or "repressor protein" it is meant a protein that exhibits the property of specifically binding to a corresponding operator sequence. An example of

repressor protein, which is not to be considered limiting in any manner is the ROS repressor, or an analog or derivative thereof as defined herein. By "ROS repressor" it is meant any ROS repressor as known within the art. These include the ROS repressor as described herein, as well as other microbial ROS repressors, for example but not limited to ROSAR (*Agrobacterium radiobacter*; Brightwell et al., (1995) Mol. Plant Microbe Interact. 8: 747-754), MucR (*Rhizobium meliloti*; Keller M et al., (1995) Mol. Plant Microbe Interact. 8: 267-277), and ROSR (*Rhizobium elti*; Bittinger et al., (1997) Mol. Plant Microbe Interact. 10: 180-186; also see Cooley et al. 1991, J. Bacteriol. 173: 2608-2616; Chou et al., 1998, Proc. Natl. Acad. Sci., 95: 5293; Archdeacon J et al. 2000, FEMS Microbiol Let. 187: 175-178; D'Souza-Ault M. R., 1993, J Bacteriol 175: 3486-3490; all of which are incorporated herein by reference). Examples of a ROS repressor, which are not to be considered limiting, are provide in Figures 1(A) to (C) and (SEQ ID NO's: 1-3 and 21). An analog, or a derivative, of a repressor protein may be any protein that exhibits the property of binding an operator sequence, for example which is not to be considered limiting in any manner, a fusion protein comprising an operator binding sequence fused to a second protein. The second protein may be any protein, including:

- a protein having an activity that regulates gene expression when bound to the operator sequence, for example but not limited to histone deacetylase, histone acetyl transferase, yeast Sin3 protein (which recruits Rpd3 (HDA complex) by binding to the DNA binding protein), Ume6, or transcriptional activators, for example but nit limited to VP16, Gal4, LexA; or
- a protein involved in protein-protein interaction, for example but not limited to chromatin remodelling proteins and HAT/HDA recruitment factors (Lusser A., Kolle D., Loidl P., 2001, Trends Plt. Sci. 6: 59-65); or
- a protein that does not directly interact with transcriptional processes but when bound to the operator sequence exhibits a property of blocking interaction of polymerase, or other factors required for transcription, with the promoter region, or migration of polymerase along a nucleic acid comprising the operator sequence, or both, blocks interaction of transcription factors with the promoter region and blocks polymerase migration.

Preferably the repressor protein or fusion protein comprises a nuclear localization signal so that the protein or fusion protein is directed to the nucleus.

5 By "codon optimization" it is meant the selection of appropriate DNA nucleotides for the synthesis of oligonucleotide building blocks, and their subsequent enzymatic assembly, of a structural gene or fragment thereof in order to approach codon usage within plants.

10 By "operator sequence" it is meant a sequence of DNA that can interact or bind with a DNA binding domain of a protein, for example, a repressor protein. An example of a repressor protein, or a DNA binding domain, that exhibits the property of binding to an operator sequence, and which is not to be considered limiting, is a ROS repressor, or the DNA binding domain of the ROS repressor, respectively. The operator sequence is preferably located in proximity of a gene of interest, either upstream of, downstream  
15 of, or within, the coding region of a gene, for example within an intron of a gene. When the repressor protein, or the DNA binding domain of the repressor, binds the operator sequence expression of the gene in operative association with the operator sequence is reduced. Preferably, the operator sequence is located in the proximity of a regulatory region that is in operative association with a gene of interest. However, the operator  
20 sequence may also be localized elsewhere within a first genetic construct to block migration of polymerase along the nucleic acid.

An operator sequence may consist of inverted repeat or palindromic sequences of a specified length. The ROS operator may comprise 9 or more nucleotide base pairs  
25 (see Figures 1 (D) and (E)) that exhibits the property of binding a DNA binding domain of a ROS repressor. A consensus sequence of a 10 base pair region including the 9 base pair DNA binding site sequence is WATDHWKMAR (SEQ ID NO: 20; Figure 1 (E)). The last nucleotide, "R", of the consensus sequence is not required for ROS binding (data not presented). Examples of operator sequences, which are not to be considered  
30 limiting in any manner, also include, as is the case with the ROS operator sequence from

the virC or virD gene promoters, a ROS operator made up of two 11bp inverted repeats separated by TTTA:

TATATTTCAATTTTATTGTAATATA (SEQ ID NO:8); or

the operator sequence of the IPT gene:

TATAATTAAAATATTAAGTGTGCGCATT (SEQ ID NO:19).

However, it is to be understood that analogs or variants of SEQ ID NO's:8, 19 and 20 may also be used providing they exhibit the property of binding a DNA binding domain, preferably a DNA binding domain of the ROS repressor. The ROS repressor has a DNA binding motif of the C<sub>2</sub>H<sub>2</sub> zinc finger configuration. In the promoter of the divergent VirC/VirD genes of *Agrobacterium tumefaciens*, ROS binds to a 9 bp inverted repeat sequence in an orientation-independent manner (Chou et al., 1998, Proc. Natl. Acad. Sci., 95: 5293). The ROS operator sequence in the *ipt* promoter also consists of a similar sequence to that in the virC/virD except that it does not form an inverted repeat (Chou et al., 1998, Proc. Natl. Acad. Sci. USA, 95: 5293). Only the first 9 bp are homologous to ROS box in virC/virD indicating that the second 9 bp sequence may not be a requisite for ROS binding. Accordingly, the use of ROS operator sequences or variants thereof that retain the ability to interact with ROS, as operator sequences to selectively control the expression of genes or nucleotide sequences of interest, is within the scope of the present invention.

By "regulatory region" or "regulatory element" it is meant a portion of nucleic acid typically, but not always, upstream of the protein coding region of a gene, which may be comprised of either DNA or RNA, or both DNA and RNA. When a regulatory region is active, and in operative association with a gene of interest, this may result in expression of the gene of interest. A regulatory element may be capable of mediating organ specificity, or controlling developmental or temporal gene activation. A "regulatory region" includes promoter elements, core promoter elements exhibiting a



basal promoter activity, elements that are inducible in response to an external stimulus, elements that mediate promoter activity such as negative regulatory elements or transcriptional enhancers. "Regulatory region", as used herein, also includes elements that are active following transcription, for example, regulatory elements that modulate gene expression such as translational and transcriptional enhancers, translational and transcriptional repressors, upstream activating sequences, and mRNA instability determinants. Several of these latter elements may be located proximal to the coding region.

In the context of this disclosure, the term "regulatory element" or "regulatory region" typically refers to a sequence of DNA, usually, but not always, upstream (5') to the coding sequence of a structural gene, which controls the expression of the coding region by providing the recognition for RNA polymerase and/or other factors required for transcription to start at a particular site. However, it is to be understood that other nucleotide sequences, located within introns, or 3' of the sequence may also contribute to the regulation of expression of a coding region of interest. An example of a regulatory element that provides for the recognition for RNA polymerase or other transcriptional factors to ensure initiation at a particular site is a promoter element. Most, but not all, eukaryotic promoter elements contain a TATA box, a conserved nucleic acid sequence comprised of adenosine and thymidine nucleotide base pairs usually situated approximately 25 base pairs upstream of a transcriptional start site. A promoter element comprises a basal promoter element, responsible for the initiation of transcription, as well as other regulatory elements (as listed above) that modify gene expression.

There are several types of regulatory regions, including those that are developmentally regulated, inducible or constitutive. A regulatory region that is developmentally regulated, or controls the differential expression of a gene under its control, is activated within certain organs or tissues of an organ at specific times during the development of that organ or tissue. However, some regulatory regions that are developmentally regulated may preferentially be active within certain organs or tissues

at specific developmental stages, they may also be active in a developmentally regulated manner, or at a basal level in other organs or tissues within the plant as well.

5 An inducible regulatory region is one that is capable of directly or indirectly  
activating transcription of one or more DNA sequences or genes in response to an  
inducer. In the absence of an inducer the DNA sequences or genes will not be  
transcribed. Typically the protein factor, that binds specifically to an inducible regulatory  
region to activate transcription, may be present in an inactive form which is then directly  
or indirectly converted to the active form by the inducer. However, the protein factor  
10 may also be absent. The inducer can be a chemical agent such as a protein, metabolite,  
growth regulator, herbicide or phenolic compound or a physiological stress imposed  
directly by heat, cold, salt, or toxic elements or indirectly through the action of a  
pathogen or disease agent such as a virus. A plant cell containing an inducible regulatory  
region may be exposed to an inducer by externally applying the inducer to the cell or  
15 plant such as by spraying, watering, heating or similar methods. Inducible regulatory  
elements may be derived from either plant or non-plant genes (e.g. Gatz, C. and Lenk,  
I.R.P.,1998, Trends Plant Sci. 3, 352-358; which is incorporated by reference).  
Examples, of potential inducible promoters include, but not limited to, tetracycline-  
inducible promoter (Gatz, C.,1997, Ann. Rev. Plant Physiol. Plant Mol. Biol. 48, 89-108;  
20 which is incorporated by reference), steroid inducible promoter (Aoyama, T. and Chua,  
N.H.,1997, Plant J. 2, 397-404; which is incorporated by reference) and ethanol-inducible  
promoter (Salter, M.G., et al, 1998, Plant Journal 16, 127-132; Caddick, M.X., et  
al,1998, Nature Biotech. 16, 177-180, which are incorporated by reference) cytokinin  
inducible IB6 and CKI1 genes (Brandstatter, I. and Kieber, J.J.,1998, Plant Cell 10, 1009-  
25 1019; Kakimoto, T., 1996, Science 274, 982-985; which are incorporated by reference)  
and the auxin inducible element, DR5 (Ulmasov, T., et al., 1997, Plant Cell 9, 1963-  
1971; which is incorporated by reference).

30 A constitutive regulatory region directs the expression of a gene throughout the  
various parts of a plant and continuously throughout plant development. Examples of  
known constitutive regulatory elements include promoters associated with the CaMV 35S

transcript. (Odell et al., 1985, *Nature*, 313: 810-812), the rice actin 1 (Zhang et al, 1991, *Plant Cell*, 3: 1155-1165), actin 2 (An *et al.*, 1996, *Plant J.*, 10: 107-121), or tms 2 (U.S. 5,428,147, which is incorporated herein by reference), and triosephosphate isomerase 1 (Xu et. al., 1994, *Plant Physiol.* 106: 459-467) genes, the maize ubiquitin 1 gene (Cormejo  
5 et al, 1993, *Plant Mol. Biol.* 29: 637-646), the *Arabidopsis* ubiquitin 1 and 6 genes (Holtorf et al, 1995, *Plant Mol. Biol.* 29: 637-646), and the tobacco translational initiation factor 4A gene (Mandel et al, 1995 *Plant Mol. Biol.* 29: 995-1004). The term  
"constitutive" as used herein does not necessarily indicate that a gene under control of the  
constitutive regulatory region is expressed at the same level in all cell types, but that the  
10 gene is expressed in a wide range of cell types even though variation in abundance is  
often observed.

The regulatory regions of the first and second nucleotide sequences denoted  
above, may be the same or different. For example, which is not to be considered limiting  
15 in any manner, the regulatory elements of the first and second genetic constructs may  
both be constitutive. In this case, each of the first and second nucleotide sequences are  
maintained in separate plants, a first and a second plant, respectively. The first nucleotide  
sequence encoding a gene of interest is expressed within the first plant. The second plant  
expresses the second nucleic acid sequence encoding a repressor protein. Crossing of  
20 the first and second plants produces a progeny that expresses the repressor protein but  
not the gene of interest. In this manner the expression of gene of interest that is required  
to maintain parent stocks may be retained within a parent plant but not expressed in a  
progeny plant. Such a cross may produce sterile offspring.

25 Alternatively, which is not to be considered limiting in any manner, either the  
second regulatory element may be active before, during, or after, the activity of the first  
regulatory element, thereby either initially repressing expression of the gene of interest  
followed by permitting the expression of the gene of interest, or, following expression  
of the gene of interest, the second regulatory element becomes active which results in the  
30 repression of the expression of the gene of interest. Similarly, the first regulatory element  
may be active before, during, or after, the activity of the second regulatory element. Other

examples, which are not to be considered limiting, include the second regulatory element being an inducible regulatory element that is activated by an external stimulus so that repression of gene expression may be controlled through the addition of an inducer. The second regulatory element may also be active during a specific developmental stage preceding, during, or following that of the activity of the first regulatory element. In this way the expression of the gene of interest may be repressed or activated as desired within a plant.

The present invention is therefore directed to one or more chimeric genetic constructs comprising a gene of interest operatively linked to a regulatory element where the regulatory element is in operative association with an operator sequence. Any exogenous gene can be used as a gene of interest and manipulated according to the present invention to result in the regulated expression of the exogenous gene. The present invention also pertains to one or more chimeric constructs comprising a regulatory element in operative association with a nucleic acid sequence encoding a repressor protein.

By "gene of interest" or "nucleotide sequence of interest" it is meant any gene or nucleotide sequence that is to be expressed within a host organism. Such a nucleotide sequence of interest may include, but is not limited to, a gene whose product has an effect on plant growth or yield, for example a plant growth regulator such as an auxin or cytokinin and their analogues, or a nucleotide sequence of interest may comprise a herbicide or a pesticide resistance gene, which are well known within the art. A gene of interest may encode an enzyme involved in the synthesis of, or in the regulation of the synthesis of, a product of interest, for example, but not limited to a protein, or an oil product. A nucleotide sequence of interest may encode an industrial enzyme, protein supplement, nutraceutical, or a value-added product for feed, food, or both feed and food use. Examples of such proteins include, but are not limited to proteases, oxidases, phytases, chitinases, invertases, lipases, cellulases, xylanases, enzymes involved in oil biosynthesis etc.

A nucleotide sequence of interest may also include a gene that encodes a pharmaceutically active protein, for example growth factors, growth regulators, antibodies, antigens, their derivatives useful for immunization or vaccination and the like. Such proteins include, but are not limited to, interleukins, insulin, G-CSF, GM-CSF, hPG-CSF, M-CSF or combinations thereof, interferons, for example, interferon- $\alpha$ , interferon- $\beta$ , interferon- $\tau$ , blood clotting factors, for example, Factor VIII, Factor IX, or tPA or combinations thereof. If the gene of interest encodes a product that is directly or indirectly toxic to the plant, then by using the method of the present invention, such toxicity may be reduced throughout the plant by selectively expressing the gene of interest within a desired tissue or at a desired stage of plant development.

A nucleotide sequence of interest may also include a gene that encodes a protein involved in regulation of transcription, for example DNA-binding proteins that act as enhancers or basal transcription factors, histone deacetylases, or histone acetyl transferases. Moreover, a nucleotide sequence of interest may be comprised of a partial sequence or a chimeric sequence of any of the above genes, in a sense or antisense orientation.

It is also contemplated that a gene of interest may be involved in the expression of a gene expression cascade, for example but not limited to a developmental cascade. In this embodiment, the gene of interest is preferably associated with a gene that is involved at an early stage within the gene cascade, for example homeotic genes. Expression of a gene of interest, for example a repressor of homeotic gene expression, represses the expression of a homeotic gene. Expression of the repressor protein within the same plant, either via crossing, induction, temporal or developmental expression of the regulatory region, as described herein, de-represses the expression of the homeotic gene thereby initiating a gene cascade. Homeotic genes are well known to one of skill in the art, and include but are not limited to, transcription factor proteins and associated regulatory regions, for example controlling sequences that bind AP2 domain containing transcription factors, for example but not limited to, APETALA2 (a regulator of meristem identity, floral organ specification, seedcoat development and floral homeotic gene

expression; Jofuku *et al.*, 1994), CCAAT box-binding transcription factors (e.g. *LEC1*; WO 98/37184; Lotan, T., et al., 1998, Cell 93, 1195-1205), or the controlling factor associated with PICKLE, a gene that produces a thickened, primary root meristem (Ogas, J., et al., 1997, Science 277, 91-94).

5

A gene of interest may also be involved in the control of transgenes across generations, or production of F1 hybrid plants with seed characteristics that would be undesirable in the parental line or progeny, for example but not limited to, oil seeds characterized as having reduced levels of sinapine biosynthesis within the oil-free meal.

10

In this case, a gene of interest may be any enzyme involved in the synthesis of one or more intermediates in sinapine biosynthesis. An example, which is to be considered non-limiting, is caffeic o-methyltransferase (Acc# AAG51676), which is involved in ferulic acid biosynthesis. Other examples of genes of interest include genes that encode proteins involved in fiber, or glucosinolate, biosynthesis, or a protein involved in the biosynthesis

15

of a phytotoxin. Phytotoxins may also be used for plant selection purposes. In this non-limiting example, a gene of interest may encode a protein that is capable of metabolizing a compound from a non-toxic form to a toxic form thereby selectively removing plants that express the gene of interest. The phytotoxic compound may be synthesized from endogenous precursors that are metabolized by the gene of interest into a toxic form, for

20

example plant growth regulators, or the phytotoxic compound may be synthesized from an exogenously applied compound that is only metabolized into a toxic compound in the presence of the gene of interest. For example, which is not to be considered limiting, the

gene of interest may comprise indole acetamide hydrolase (IAH), that converts exogenously applied indole acetamide (IAM) or naphthaline acetamide (NAM), to indole acetic acid (IAA), or naphthaline acetic acid (NAA), respectively. Over-synthesis of IAA

25

or NAA is toxic to a plant, however, in the absence of IAH, the applied IAM or NAM is non-toxic. Similarly, the gene of interest may encode a protein involved in herbicide resistance, for example, but not limited to, phosphinothricin acetyl transferase, wherein, in the absence of the gene encoding the transferase, application of phosphinothricin, the

30

toxic compound (herbicide) results in plant death. Other gene of interest that encode

lethal or conditionally lethal products may be found in WO 00/37660 (which is incorporated herein by reference).

5       The gene of interest or the nucleotide sequence of interest may be expressed in suitable plant hosts which are transformed by the nucleotide sequences, or nucleic acid molecules, or genetic constructs, or vectors of the present invention. Examples of suitable hosts include, but are not limited to, agricultural crops including canola, *Brassica* spp., maize, tobacco, alfalfa, rice, soybean, wheat, barley, sunflower, and cotton.

10       The one or more chimeric genetic constructs of the present invention can further comprise a 3' untranslated region. A 3' untranslated region refers to that portion of a gene comprising a DNA segment that contains a polyadenylation signal and any other regulatory signals capable of effecting mRNA processing or gene expression. The polyadenylation signal is usually characterized by effecting the addition of polyadenylic acid tracks to the 3' end of the mRNA precursor. Polyadenylation signals are commonly  
15       recognized by the presence of homology to the canonical form 5' AATAAA-3' although variations are not uncommon. One or more of the chimeric genetic constructs of the present invention can also include further enhancers, either translation or transcription enhancers, as may be required. These enhancer regions are well known to persons skilled  
20       in the art, and can include the ATG initiation codon and adjacent sequences. The initiation codon must be in phase with the reading frame of the coding sequence to ensure translation of the entire sequence.

25       Examples of suitable 3' regions are the 3' transcribed non-translated regions containing a polyadenylation signal of *Agrobacterium* tumor inducing (Ti) plasmid genes, such as the nopaline synthase (Nos gene) and plant genes such as the soybean storage protein genes and the small subunit of the ribulose-1, 5-bisphosphate carboxylase (ssRUBISCO) gene.

30       To aid in identification of transformed plant cells, the constructs of this invention may be further manipulated to include plant selectable markers. Useful selectable

markers include enzymes which provide for resistance to chemicals such as an antibiotic for example, gentamycin, hygromycin, kanamycin, or herbicides such as phosphinothrycin, glyphosate, chlorosulfuron, and the like. Similarly, enzymes providing for production of a compound identifiable by colour change such as GUS (β-glucuronidase), or luminescence, such as luciferase or GFP, are useful.

Also considered part of this invention are transgenic plants containing the chimeric gene construct of the present invention. However, it is to be understood that the chimeric gene constructs of the present invention may also be combined with gene of interest for expression within a range of plant hosts.

Methods of regenerating whole plants from plant cells are also known in the art. In general, transformed plant cells are cultured in an appropriate medium, which may contain selective agents such as antibiotics, where selectable markers are used to facilitate identification of transformed plant cells. Once callus forms, shoot formation can be encouraged by employing the appropriate plant hormones in accordance with known methods and the shoots transferred to rooting medium for regeneration of plants. The plants may then be used to establish repetitive generations, either from seeds or using vegetative propagation techniques. Transgenic plants can also be generated without using tissue cultures (for example, Clough and Bent, 1998)

The constructs of the present invention can be introduced into plant cells using Ti plasmids, Ri plasmids, plant virus vectors, direct DNA transformation, micro-injection, electroporation, etc. For reviews of such techniques see for example Weissbach and Weissbach, *Methods for Plant Molecular Biology*, Academy Press, New York VIII, pp. 421-463 (1988); Geierman and Corey, *Plant Molecular Biology*, 2d Ed. (1988); and Miki and Iyer, *Fundamentals of Gene Transfer in Plants*. In *Plant Metabolism*, 2d Ed. DT. Dennis, DH Turpin, DD Lefebvre, DB Layzell (eds), Addison Wesley, Langmans Ltd. London, pp. 561-579 (1997); Clough and Bent (1998)). The present invention further includes a suitable vector comprising the chimeric gene construct.



An "analogue" or "derivative" includes any substitution, deletion, or addition to the nucleotide or amino acid sequence of the repressor protein, for example but not limited to, the ROS repressor, provided that the analogue or derivative thereof, maintains the property of binding or associating with the operator sequence, ROS repressor activity, or both. Preferably, the repressor protein, or an analogue or derivative thereof exhibits the property of binding an operator sequence, and exhibits the property of repressing the expression of a gene in operative association with the operator sequence.

The DNA sequences of the present invention include the DNA sequences of SEQ ID NO: 1, 2 and 3 (native or wild-type ROS repressor, synthetic ROS repressor, and a composite or consensus ROS repressor; also see Figures 1(B) and-(C)) derivatives, and fragments thereof, as well as analogues of, or nucleic acid sequences that are substantially homologous to, and that exhibit greater than 80% similarity with, the nucleic acid sequence as defined in SEQ ID NO: 2 or 3. If a fragment of a ROS repressor is used, the fragment is at least of about 54 nucleotides in length in order to cover the zinc finger domain (from 249 to 303). Preferably, the fragment is from about 54 to about 150 nucleotides in length, more preferably from about 54 to about 80 nucleotides in length.

Sequences that exhibit greater than 80% similarity, may be determined by use of the BLAST algorithm (GenBank: [www.ncbi.nlm.nih.gov/cgi-bin/BLAST/](http://www.ncbi.nlm.nih.gov/cgi-bin/BLAST/)), using default parameters (Program: blastn; Database: nr; Expect 10; filter: low complexity; Alignment: pairwise; Word size:11). Analogs, or derivatives thereof, also include those DNA sequences which hybridize under stringent hybridization conditions (see Maniatis et al., in Molecular Cloning (A Laboratory Manual), Cold Spring Harbor Laboratory, 1982, p. 387-389) to any one of the DNA sequences of SEQ ID NO: 1, 2 or 3 provided that the sequences exhibit the property of binding an operator sequence (operator binding activity), or maintain the property of repressing the expression of a gene in operative association with the operator sequence. An example of one such stringent hybridization conditions may be hybridization with a suitable probe, for example but not limited to, a [ $\alpha$ -<sup>32</sup>P]dATP labelled probe for 16-20 hrs at 65°C in 7% SDS, 1mM EDTA, 0.5M Na<sub>2</sub>HPO<sub>4</sub>, pH 7.2. Followed by washing in 5% SDS, 1mM EDTA 40mM Na<sub>2</sub>HPO<sub>4</sub>, pH

7.2 for 30 min followed by washing in 1% SDS, 1mM EDTA 40mM Na<sub>2</sub>HPO<sub>4</sub>, pH 7.2 for 30 min. Washing in this buffer may be repeated to reduce background. An example of an analog or a derivative of the ROS repressor, which is not to be considered limiting in any manner, includes the ROS operator binding sequence fused to a second protein to produce a fusion protein, providing that the fusion protein exhibits ROS operator sequence binding activity.

The second protein that is fused to the DNA binding sequence, may be any protein, including a protein having an activity that regulates gene expression when bound to the operator sequence, for example but not limited to histone deacetylase, histone acetyl transferase, a protein involved in protein-protein interaction, or a protein that does not directly interact with transcriptional processes, but that exhibits a characteristic of steric hindrance, for example, interfering with the association of polymerase or other transcription factor within the promoter region, or by blocking migration of polymerase along a nucleic acid.

The present invention is further directed to one or more nucleotide constructs comprising a nucleotide sequence of interest operatively linked to a regulatory region that is modified to contain one or more operator sequences, for example, but not limited to, one or more ROS operator sequences (see Figures 3, 4, or 5). As shown in Figure 3 an operator sequence may be placed downstream (Figure 3(A)), upstream (Figure 3(B)), or upstream and downstream (Figure 3(C)) of the TATA box within a regulatory region. The operator sequences may be placed within a promoter region as single binding elements or as tandem repeats (see Figure 5(B)). Furthermore, as shown in Figure 4(B)), tandem repeats of an operator sequence can be placed downstream of the entire promoter or regulatory region and upstream of the gene or nucleotide sequence of interest. An operator sequence, or repeats of an operator sequence may also be positioned within untranslated or translated leader sequences (if positioned in-frame), introns of a gene, or within an ORF of a gene, if inserted in-frame. Any gene or nucleotide sequence may be used as the gene or nucleotide sequence of interest and be selectively targeted for regulation of gene expression according to the present invention.

The repressor protein that is produced from the second nucleotide sequence, for example but not limited to a ROS repressor, can bind to operator sequences contained within the regulatory region of the first nucleotide sequence and thereby specifically and selectively repress transcription of the gene of interest. Preferably, the first nucleotide sequence and the second nucleotide sequence are chromosomally integrated into a plant or plant cell. The two nucleotide sequences may be integrated into two different genetic loci of a plant or plant cell, or the two nucleotide sequences may be integrated into a singular genetic locus of a plant or plant cell.

The ROS transcription factor (ROS repressor, Figure 1(A); SEQ ID NO:3), for example, of *Agrobacterium tumefaciens* (SEQ ID NO's:1 and 21, nucleic acid and amino acid sequence, respectively) has a DNA binding motif (see bolded amino acids, Figure 1(C)) of the C<sub>2</sub>H<sub>2</sub> zinc finger configuration (Chou et al., 1998, Proc. Natl. Acad. Sci., 95: 5293). Zinc finger DNA binding proteins represent a significant portion of transcription factors in eukaryotes, but are rare in prokaryotes. The zinc finger ROS protein varies from its counterparts in eukaryotes in two aspects:

1. Unlike most eukaryotic zinc finger proteins, which contain multiple zinc finger motifs, the ROS repressor has only one such motif.
2. There are 9 amino acid residues making up the peptide loop spacing the zinc finger motif in the ROS repressor as compared to the 12 amino acids that make up the loops of zinc fingers of eukaryotic proteins.

These two characteristics of the ROS zinc finger motif, and possibly, the small size of the ROS repressor (~15.5 kDa) provide structural uniqueness and molecular flexibility and that make the ROS repressor, or analogs thereof, a suitable candidate as a transcription factor for regulation of gene expression in plants. However, it is to be understood that larger size chimeric proteins comprising a ROS operator binding domain may also be used as described herein.

The ROS repressor is encoded by a nucleotide sequence of bacterial origin and, as such the nucleotide sequence may be optimised, for example, by changing its codons to favour plant codon usage (e.g. SEQ ID NO:2), by attaching a nucleotide sequence

encoding a nuclear localisation signal, for example but not limited to SV40 localization signal (see Robbins *et al.*, 1991, *Cell*, 64: 615-623; Rizzo, P., Di Resta, I., Powers, A., Ratner, H. and Carbone, M. 1991, *Cancer Res.* 59 (24), 6103-6108; which are incorporated herein by reference) in order to improve the efficiency of ROS transport to the plant nucleus to facilitate the interaction with its respective operator, or both optimizing plant codon usage and fusing a nuclear localization signal to the ROS repressor nucleic acid sequence. Other possible nuclear localization signals that may be used include but are not limited to those listed in Table 1:

**Table 1: nuclear localization signals**

<b>Nuclear Protein</b>	<b>Organism</b>	<b>NLS</b>	<b>Ref</b>
AGAMOUS	A	RienttnrqvtfcKRR	1
TGA-1A	T	RRlaqnreaaRKsRIKK	2
TGA-1B	T	KKRaRlvnresaqlsRqRKK	2
O2 NLS B	M	RKRKesnresaRRsRyRK	3
NIa	V	KKnqkhklkm-32aa-KRK	4
Nucleoplasmin	X	KRpaaatkkagqaKKKKI	5
NO38	X	KRIapdsaskvpRKKtR	5
N1/N2	X	KRKteesplKdKdaKK	5
Glucocorticoid			
receptor	M,R	RKclqagmnleaRKtKK	5
$\alpha$ receptor	H	RKclqagmnleaRKtKK	5
$\beta$ receptor	H	RKclqagmnleaRKtKK	5
Progesterone receptor	C,H,Ra	RKccqagmvlggRKfKK	5
Androgen receptor	H	RKcyeagmtlgaRKlKK	5
p53	C	RRcfevrycacpgRdRK	5

<sup>+</sup>A, *Arabidopsis*; X, *Xenopus*; M, mouse; R, rat; Ra, rabbit; H, human; C, chicken; T, tobacco; M, maize; V, potyvirus.

References:

- 1, Yanovsky *et al.*, 1990, Nature, 346: 35-39
  - 2, van der Krol and Chua, 1991, Plant Cell, 3: 667-675
  - 3, Varagona *et al.*, 1992, Plant Cell, 4: 1213-1227
  - 4, Carrington *et al.*, 1991, Plant Cell, 3: 953-962
  - 5, Robbins *et al.*, 1991, Cell, 64: 615-623
- 

The fusion of a nuclear localization signal to the repressor protein or fusion protein facilitates migration of the repressor, or fusion, protein into the nucleus. Without wishing to be bound by theory, reduced levels of repressor or fusion proteins elsewhere within the cell may be important when the repressor or fusion protein may bind analogue operator sequences within other organelles, for example within the mitochondrion or chloroplast. Furthermore, the use of a nuclear localization signal may permit the use of a less active promoter or regulatory region to drive the expression of the repressor, or fusion, protein while ensuring that the concentration of the expressed protein remains at a desired level within the nucleus, and that the concentration of the protein is reduced elsewhere in the cell.

The nuclear localization signal may be fused to the N, C, or both the N and C terminus of the ROS protein. Furthermore, the nuclear localization signal may be fused within the coding region of the gene, provided that the activity of the protein is retained. Preferably, the nuclear localization signal is fused to the carboxy-terminus of the protein or fusion protein. The nucleotide sequence, depicted in Figure 1(B) or SEQ ID NO:2, consisting of the fusion of the modified nucleotide sequence of the protein coding region of ROS with the nucleotide sequence encoding the nuclear localization signal is designated as "synthetic ROS". Thus, analogues of the nucleotide sequence encoding ROS repressor, or the amino acid sequence of the ROS repressor, are within the scope of the present invention.

In order to optimize expression levels and transgene protein production of a repressor protein, for example the ROS repressor, the nucleic acid sequence of the ROS

repressor was examined and the coding region modified to optimize for expression of the gene in plants. A procedure similar to that outlined by Sardana et al. (Plant Cell Reports 15:677-681; 1996) may also be used. A table of codon usage from highly expressed genes of dicotyledonous plants was compiled using the data of Murray et al. (Nuc Acids Res. 17:477-498; 1989). An example of a synthetic ROS repressor gene comprising codons optimized for expression within plants is shown in Figure 1(B). However, it is to be understood that other base pair combinations may be used for the preparation of a synthetic ROS repressor gene, for example SEQ ID NO:3, using the methods as described herein in order to optimize ROS repressor expression within a plant.

Assembly of the synthetic ROS repressor gene of this invention is performed using standard technology known in the art. The gene may be assembled enzymatically, within a DNA vector, for example using PCR, or synthesised from chemically synthesized oligonucleotide duplex segments. The synthetic gene is then introduced into a plant using methods known in the art. Expression of the gene may be determined using methods known within the art, for example Northern analysis, Western analysis, or ELISA.

The present invention also pertains to the regulation of gene expression in plants using the ROS repressor protein, whereby the ROS repressor is used as a regulatory switch to repress the expression of selected genes or nucleotide sequences of interest. The repression of the expression of a gene of interest may be accomplished by transforming the plant with two constructs:

1. A first genetic construct comprising a gene or nucleotide sequence of interest operatively associated with a regulatory region containing at least one operator sequence that can interact with the ROS repressor.

2. A second genetic construct comprising an appropriate regulatory region operatively linked to a nucleotide sequence that encodes the ROS repressor.

The first and second genetic constructs may be inserted into a plant in separate vectors, each of which may be introduced into a plant via co-transformation sequentially, or at the same time, or introduced into a plant by crossing plants expressing either the first or second genetic construct, or both genetic constructs may reside within one vector, and be introduced within a plant at the same time.

Preferably, the protein coding region of the nucleotide sequence encoding the ROS repressor is modified to favour plant codon usage. Furthermore, it is preferred that the nucleotide sequence is operatively linked with a nucleotide sequence encoding a nuclear localisation signal. Expression of both constructs within the same plant will result in a repression of the expression of the gene of interest as mediated by an interaction of the ROS repressor with a ROS operator sequence contained within the regulatory region of the first genetic construct.

Schematic representations of constructs capable of expressing synthetic ROS or wild type ROS are shown in Figure 2(A; wild type ROS) and Figures 2(B)-(D; synthetic ROS). Southern analysis (Figure 6 (B)) of *Arabidopsis* plants that are transformed with constructs comprising the second nucleic acid sequence of the present invention, expressing ROS repressor protein, indicates that both the wild type ROS and the synthetic ROS are integrated into the chromosome of *Arabidopsis*. Western blots shown in Figure 7 demonstrate that both native ROS and synthetic ROS may be expressed within plants.

Similarly, stable integration and expression of the first nucleotide sequence of the present invention comprising a gene of interest, in operative association with a regulatory region which is in operative association with an operator sequence is seen in Figure 6 (A) (Southern analysis) and Figure 8 (GUS expression).

Crossing plants expressing the first nucleotide sequence, comprising a gene of interest, for example but not limited to GUS, and the second nucleotide sequence encoding ROS repressor, either native or synthetic ROS, exhibit reduced expression of

the gene of interest, in this case GUS. Results of a cross between a transgenic line expressing synthetic ROS (ROS parent) and a gene of interest, for example, but not limited to GUS (GUS parent), are presented in Figure 9 and demonstrate ROS repression of a gene of interest. The results in Figure 9A demonstrate that GUS activity is detected in the GUS parent but not in the ROS parent (does not comprise the GUS construct), or in the progeny of the cross between the ROS and GUS parent. The parent plants each expressed either GUS or ROS RNA as expected (Figure 9B), yet no GUS RNA was detected in the progeny arising from a cross between the ROS and GUS parents. Southern analysis of the progeny of the cross between the GUS and ROS parents indicates that the progeny plant from the cross between the ROS and GUS parent comprised genes encoding both GUS and ROS (Figure 9C).

These data demonstrate that expression of a gene of interest can be controlled using the repressor mediated system as described herein.

The above description is not intended to limit the claimed invention in any manner, furthermore, the discussed combination of features might not be absolutely necessary for the inventive solution.

The present invention will be further illustrated in the following examples. However it is to be understood that these examples are for illustrative purposes only, and should not be used to limit the scope of the present invention in any manner.

## Examples

## Materials and Methods

### Plant Material

Wild type *Arabidopsis thaliana*, ecotype Columbia, seeds were germinated on RediEarth (W.R. Grace & Co., Ajax, On) soil in pots covered with window screens under



green house conditions (~25°C, 16 hr light). Emerging bolts were cut back to encourage further bolting. Plants were used for transformation once multiple secondary bolts had been generated.

## 5 Plant Transformation

Plant transformation was carried out according to the floral dip procedure described in Clough and Bent (1998, Plant J., 16, 735). Essentially, *Agrobacterium tumefaciens* transformed with the construct of interest (using standard methods as known  
10 in the art) was grown overnight in a 100ml Luria-Bertani Broth (10 g/L NaCl, 10 g/L tryptone, 5 g/L yeast extract) containing 50 ug/ml kanamycin. The cell suspension culture was centrifuged at 3000 X g for 15 min. The pellet was resuspended in 1L of the transformation buffer (sucrose (5%), Silwet L77 (0.05%)(Loveland Industries, Greeley, Co.)). The above-ground parts of the *Arabidopsis* plants were dipped into the  
15 *Agrobacterium* suspension for ~1 min and the plants were then transferred to the greenhouse. The entire transformation process was repeated twice more at two day intervals. Plants were grown to maturity and seeds collected. To select for transformants, seeds were surface sterilized by washing in 0.05% Tween 20 for 5 minutes, with 95% ethanol for 5 min, and then with a solution containing sodium hypochlorite (1.575%) and  
20 Tween 20 (0.05%) for 10 min followed by 5 washings in sterile water. Sterile seeds were plated onto either Pete Lite medium (20-20-20 Peter's Professional Pete Lite fertilizer (Scott, Marysville, OH) (0.762 g/l), agar (0.7%), kanamycin (50 ug/ml), pH 5.5) or MS medium (MS salts (0.5X)(Sigma), B5 vitamins (1X), agar (0.7%), kanamycin (50 ug/ml) pH 5.7). Plates were incubated at 20°C, 16hr light/ 8 hr dark in a growth room. After  
25 approximately two weeks, seedlings possessing green primary leaves were transferred to soil for further screening and analysis.

### **Example 1: Optimization of ROS protein coding region.**

30 The *ros* nucleotide sequence is derived from *Agrobacterium tumefaciens* (SEQ ID NO:1; Figure 1A). Analysis of the protein coding region of the *ros* nucleotide

sequence indicates that the codon usage may be altered to better conform to plant translational machinery. The protein coding region of the *ros* nucleotide sequence was therefore modified to optimize expression in plants (SEQ ID NO:2; Figure 1B). The nucleic acid sequence of the ROS repressor was examined and the coding region modified to optimize for expression of the gene in plants, using a procedure similar to that outlined by Sardana et al. (Plant Cell Reports 15:677-681; 1996). A table of codon usage from highly expressed genes of dicotyledonous plants was compiled using the data of Murray et al. (Nuc Acids Res. 17:477-498; 1989). The *ros* nucleotide sequence was also modified (SEQ ID NO:2; Figure 1B) to ensure localization of the ROS repressor to the nucleus of plant cells, by adding a SV40 nuclear localization signal (Rizzo,P., Di Resta,I., Powers,A., Ratner,H. and Carbone,M.. Cancer Res. 59 (24), 6103-6108 (1999; The nuclear localization signal resides at amino acid positions 126-132; accession number AAF28270).

The *ros* gene is cloned from *Agrobacterium tumefaciens* by PCR. The nucleotide sequence encoding the ROS protein is expressed in, and purified from, *E. coli*, and the ROS protein used to generate an anti-ROS antiserum in rabbits using standard methods (Maniatis et al.).

## **Example 2: Constructs that express synthetic ROS repressor, or wild type ROS repressor and preparation of repressor lines.**

The protein coding region of the *ros* gene is modified to favour *Arabidopsis thaliana* and *Brassica napus* codon usage, and in some constructs, to incorporate a nucleotide sequence encoding a nuclear localization signal at its carboxy terminus as described below. A modified *ros* nucleotide sequence comprising optimized codons and the nuclear localization signal is referred to as "synthetic ROS". In this example, the ROS coding portion of the synthetic ROS nucleotide sequence is designed to encode the same protein as the wild type bacterial *ros* nucleotide sequence, while optimizing codon usage in plants or plant cells.

**p74-107:** Construct for The Expression of The Wild Type ROS Driven by The CaMV 35S Promoter (Figure 2(A)).

The protein coding region of the wild type ROS gene is amplified by PCR using total genomic DNA of *Agrobacterium tumefaciens* 33970 and the following two primers with built-in *Bam*HI (G GAT CC) and *Hind*III (A AGC TT) sites:

Sense primer: 5- GCG GAT CCG ATG ACG GAA ACT GCA TAC-3' (SEQ ID NO:4)

Anti-sense primer: 5'-GCA AGC TTC AAC GGT TCG CCT TGC G-3' (SEQ ID NO:5).

The PCR product, which lacks any nuclear localization signal, is cloned into the *Bam*HI/*Hind*III sites of the pGEX vector (Pharmacia), excised from pGEX as a *Xho*I/*Bam*HI fragment, and the *Xho* I site blunt-ended using Klenow. The resulting fragment is cloned into the *Bam*HI/*Eco*ICR1 sites of pBI121 (Clontech, Palo Alto, California).

**p74-313:** Construct for The Expression of The Synthetic ROS Driven by The CaMV 35S Promoter (Figure 2(B)).

The ORF of the ROS repressor is re-synthesized to favor plant codon usage as outlined above, and to incorporate a SV40 nuclear localization signal, PKKKRKV, at its carboxy terminus. The re-synthesized ROS is cloned into the *Bam*HI-*Sac*I sites of pUC19, and subcloned into pBI121 as a *Bam*HI/*Sst*I fragment replacing the GUS ORF in this vector.

**p74-108:** Construct for The Expression of The Synthetic ROS Repressor Driven by the *tms2* Promoter (Figure 2(C)).

The *tms2* promoter is PCR amplified from genomic DNA of *Agrobacterium tumefaciens* 33970 using the following two primers:

sense primer: 5'-TGC GGA TGC ATA AGC TTG CTG ACA TTG CTA GAA AAG-3'  
(SEQ ID NO:6)

anti-sense primer: 5'-CGG GGA TCC TTT CAG GGC CAT TTC AG -3' (SEQ ID  
NO:7)

The 352 bp PCR fragment is cloned into the EcoRV site of pBluescript, and excised from pBluescript as a HindIII/BamHI fragment, and sub-cloned into the HindIII/BamHI sites of p74-313, see below, replacing the CaMV 35S promoter.

**p74-101: Construct for The Expression of The Synthetic ROS Driven by The Actin2 Promoter (Figure 2(D)).**

The Actin2 promoter (An *et al.*, 1996, *Plant J.*, 10: 107-121) is PCR amplified from genomic DNA of *Arabidopsis thaliana* ecotype Columbia as described in 74-501 (see below) and cloned into pGEM-T-Easy. The 1.2 kbp HindIII/SpeI fragment of the Actin2 promoter is then cloned into p74-313 (see below) as a HindIII/XbaI fragment replacing the CaMV 35S promoter.

The various constructs are introduced into *Arabidopsis*, as described above, and transgenic plants are generated. Transformed plants are verified using PCR or Southern analysis. Figure 6(B) show Southern analysis of transgenic plants comprising a second genetic construct, for example, p74-101 (actin2-synthetic ROS; Figure 2(D)).

## **Western blot analysis of repressor transgenic lines**

The expression of ROS in the repressor lines is assessed by Western blot analysis using a ROS polyclonal antibody. Several lines show high levels of ROS expression. These included plants expressing both the wild type ROS (without any nuclear localization signal) as well as those expressing the synthetic ROS nucleic acid sequences.

Total plant protein extracts are analyzed for the expression of the ROS protein using a polyclonal rabbit anti-ROS antibody. Chemiluminescent detection of antigen-antibody complexes is carried out with goat anti-rabbit IgG secondary antibody conjugated to horseradish peroxidase-conjugated (from Bio-Rad Laboratories) in conjunction with ECL detection reagent (from Amersham Pharmacia Biotech).

Levels of ROS protein, both wild type ROS (WTROS), for example p74-107 (35S-WTROS; Figure 2(A)), and synthetic ROS, for example p74-101 (actin2-synROS; Figure 2(D)), produced in the transgenic plants is determined by Western blot analysis using a ROS polyclonal antibody (Figure 7).

Representative lines showing various levels of expression were used as a source of pollen for pollination of reporter lines containing single inserts.

**Example 3: Constructs placing a gene of interest under transcriptional control of regulatory regions that have been modified to contain ROS operator sites, and preparation of reporter lines.**

**p74-315: Construct for The Expression of GUS Gene Driven by a CaMV 35S Promoter Containing a ROS Operator Downstream of TATA Box (Figure 3(A)).**

The BamHI-EcoRV fragment of CaMV 35S promoter in pBI121 is cut out and replaced with a similar synthesized DNA fragment in which the 25 bp immediately downstream of the TATA box were replaced with the ROS operator sequence:

TATATTTCAATTTTATTGTAATATA (SEQ ID NO:8).

Two complementary oligos, ROS-OPDS (SEQ ID NO:9) and ROS-OPDA (SEQ ID NO:10), with built-in BamHI-EcoRV ends, and spanning the BamHI-EcoRV region of CaMV35S, in which the 25 bp immediately downstream of the TATA box are replaced

with the ROS operator sequence (SEQ ID NO:8), are annealed together and then ligated into the BamHI-EcoRV sites of CaMV35S.

5 ROS-OPDS: 5'-ATC TCC ACT GAC GTA AGG GAT GAC GCA CAA TCC CAC  
TAT CCT TCG CAA GAC CCT TCC TCT ATA TAA TAT ATT TCA  
ATT TTA TTG TAA TAT AAC ACG GGG GAC TCT AGA G-3' (SEQ  
ID NO:9)

10 ROS-OPDA: 5'- G ATC CTC TAG AGT CCC CCG TGT TAT ATT ACA ATA AAA  
TTG AAA TAT ATT ATA TAG AGG AAG GGT CTT GCG AAG GAT  
AGT GGG ATT GTG CGT CAT CCC TTA CGT CAG TGG AGA T-3'  
(SEQ ID NO:10)

15 The p74-315 sequence from the EcoRV site (GAT ATC) to the first codon (ATG) of  
GUS is shown below (TATA box - lower case in bold; the synthetic ROS sequence - bold  
caps; a transcription start site - ACA, bold italics; BamHI site - GGA TCC; and the first  
of GUS, ATG, in italics; are also indicated):

20 5'-GAT ATC TCC ACT GAC GTA AGG GAT GAC GCA CAA TCC CAC TAT CCT  
TCG CAA GAC CCT TCC TCT **ata ta** TAT ATT TCA ATT TTA TTG TAA TAT  
**AAC** ACG GGG GAC TCT AGA GGA TCC CCG GGT GGT CAG TCC CTT *ATG*-3'  
(SEQ ID NO:22)

25 p74-316: Construct for The Expression of GUS Driven by a CaMV 35S Promoter  
Containing a ROS Operator Upstream of TATA Box (Figure 3(B)).

30 The BamHI-EcoRV fragment of CaMV 35S promoter in pBI121 is cut out and  
replaced with a similar synthesized DNA fragment in which the 25 bp immediately  
upstream of the TATA box are replaced with the ROS operator sequence (SEQ ID NO:8).  
Two complementary oligos, ROS-OPUS (SEQ ID NO:11) and ROS-OPUA (SEQ ID  
NO:12), with built-in BamHI-EcoRV ends, and spanning the BamHI-EcoRV region of

CaMV35S, in which the 25 bp immediately upstream of the TATA box were replaced with a ROS operator sequence (SEQ ID NO:8), are annealed together and then ligated into the BamHI-EcoRV sites of CaMV35S.

5 ROS-OPUS: 5'-ATC TCC ACT GAC GTA AGG GAT GAC GCA CAA TCT ATA  
TTT CAA TTT TAT TGT AAT ATA CTA TAT AAG GAA GTT CAT  
TTC ATT TGG AGA GAA CAC GGG GGA CTC TAG AG -3' (SEQ ID  
NO:11)

10 ROS-OPUA: 5'- G ATC CTC TAG AGT CCC CCG TGT TCT CTC CAA ATG AAA  
TGA ACT TCC TTA TAT AGT ATA TTA CAA TAA AAT TGA AAT  
ATA GAT TGT GCG TCA TCC CTT ACG TCA GTG GAG AT-3'  
(SEQ ID NO:12)

15 The p74-316 sequence from the EcoRV site (GAT ATC) to the first codon (ATG) of  
GUS is shown below (TATA box - lower case in bold; the synthetic ROS sequence - bold  
caps; a transcription start site - ACA, bold italics; BamHI site - GGA TCC; the first  
codon of GUS, ATG -italics, are also indicated):

20 5'-GAT ATC TCC ACT GAC GTA AGG GAT GAC GCA CAA TCT ATA TTT CAA  
TTT TAT TGT AAT ATA Cta tat aAG GAA GTT CAT TTC ATT TGG AGA GAA  
CAC GGG GGA CTC TAG AGG ATC CCC GGG TGG TCA GTC CCT TAT G-3'  
(SEQ ID NO:23)

25 p74-309: Construct for The Expression of GUS Driven by a CaMV 35S Promoter  
Containing ROS Operators Upstream and Downstream of TATA Box (Figure  
3(C)).

30 The BamHI-EcoRV fragment of CaMV 35S promoter in pBI121 is cut out and  
replaced with a similar synthesized DNA fragment in which the 25 bp immediately  
upstream and downstream of the TATA box were replaced with two ROS operator

sequences (SEQ ID NO:8). Two complementary oligos, ROS-OPPS (SEQ ID NO:13) and ROSOPPA (SEQ ID NO:14), with built-in BamHI-EcoRV ends, and spanning the BamHI-EcoRV region of CaMV35S, in which the 25 bp immediately upstream and downstream of the TATA box are replaced with two ROS operator sequences, each comprising the sequence of SEQ ID NO:8 (in italics, below), are annealed together and ligated into the BamHI-EcoRV sites of CaMV35S.

ROS-OPPS: 5'-ATC TCC ACT GAC GTA AGG GAT GAC GCA CAA TCT *ATA TTT CAA TTT TAT TGT AAT ATA* CTA TAT AAT ATA TTT CAA TTT TAT TGT AAT ATA ACA CGG GGG ACT CTA GAG-3' (SEQ ID NO:13)

ROS-OPPA: 5'-G ATC CTC TAG AGT CCC CCG TGT TAT ATT ACA ATA AAA TTG AAA TAT ATT ATA TAG *TAT ATT ACA ATA AAA TTG AAA TAT AGA* TTG TGC GTC ATC CCT TAC GTC AGT GGA GAT-3' (SEQ ID NO:14)

The p74-309 sequence from the EcoRV site (GAT ATC) to the first codon (ATG) of GUS is shown below (TATA box - lower case in bold; two synthetic ROS sequence - bold caps; a transcription start site - ACA, bold italics; BamHI site - GGA TCC; the first codon of GUS, ATG -italics, are also indicated):

5'-GAT ATC TCC ACT GAC GTA AGG GAT GAC GCA CAA TCT ATA TTT CAA TTT TAT TGT AAT ATA Cta tat aAT ATA TTT CAA TTT TAT TGT AAT ATA ACA CGG GGG ACT CTA GAG GAT CCC CGG GTG GTC AGT CCC TTA TG-3' (SEQ ID NO:24)

**p76-508: Construct for The Expression of The GUS Gene Driven by the tms2 Promoter Containing a ROS Operator (Figure 4(B)).**

The tms2 promoter is PCR amplified from genomic DNA of *Agrobacterium tumefaciens* 33970 using the following primers:



sense primer: 5'-TGC GGA TGC ATA AGC TTG CTG ACA TTG CTA GAA AAG-  
3' (SEQ ID NO:6)

anti-sense primer: 5'-CGG GGA TCC TTT CAG GGC CAT TTC AG- 3'(SEQ ID  
NO:7)

5

The 352 bp PCR fragment is cloned into the EcoRV site of pBluescript, and sub-cloned  
into pGEM-7Zf(+). Two complementary oligos, ROS-OP1 (SEQ ID NO:15) and ROS-  
OP2 (SEQ ID NO:16), containing two ROS operators (in italics, below), are annealed  
together and cloned into pGEM-7Zf(+) as a BamHI/ClaI fragment at the 3' end of the  
10 *tms2* promoter. This promoter/operator fragment is then sub-cloned into pBI121 as a  
*HindIII/XbaI* fragment, replacing the CaMV 35S promoter fragment.

ROS-OP1: 5'-GAT CCT ATA TTT CAA TTT TAT TGT AAT ATA GCT *ATA TTT*  
*CAA TTT TAT TGT AAT ATA* AT-3' (SEQ ID NO:15)

15

ROS-P2: 5'-CGA TTA TAT TAC AAT AAA ATT GAA ATA TAG *CTA TAT TAC*  
*AAT AAA ATT GAA ATA TAG*-3'(SEQ ID NO:16).

20 As a control, p76-507 comprising a *tms2* promoter (without any operator  
sequence) fused to GUS (Figure 4(A)), is also prepared.

**p74-501:** Construct for The Expression of The GUS Gene Driven by The Actin2  
Promoter Containing a ROS operator (Figure 5(B)).

25

The Actin2 promoter is PCR amplified from genomic DNA of *Arabidopsis*  
*thaliana* ecotype Columbia using the following primers:

Sense primer: 5'- AAG CTT ATG TAT GCA AGA GTC AGC-3'(SEQ ID NO:17)

SpeI

30 Anti-sense primer: 5'- TTG ACT AGT ATC AGC CTC AGC CAT-3'(SEQ ID  
NO:18)

The PCR fragment is cloned into pGEM-T-Easy. Two complementary oligos, ROS-OP1 (SEQ ID NO:15) and ROS-OP2 (SEQ ID NO:16), with built-in BamHI and ClaI sites, and containing two ROS operators, are annealed together and inserted into the Actin2 promoter at the BglII/Cla I sites replacing the BglII/ClaI fragment. This modified promoter is inserted into pBI121 vector as a HindIII/BamHI fragment.

**p74-118 Construct for The Expression of GUS Driven by a CaMV 35S Promoter Containing three ROS Operators Downstream of TATA Box (Figure 5(C)).**

The BamHI-EcoRV fragment of CaMV 35S promoter in pBI121 is cut out and replaced with a similar synthesized DNA fragment in which a region downstream of the TATA box was replaced with three ROS operator sequences (SEQ ID NO:25). The first of the three synthetic ROS operator sequences is positioned immediately of the TAT box, the other two ROS operator sequence are located downstream of the transcriptional start site (ACA). Two complementary oligos with built-in BamHI-EcoRV ends were prepared as describe above for the other constructs were annealed together and ligated into the BamHI-EcoRV sites of CaMV35S.

The p74-118 sequence from the EcoRV site (GAT ATC) to the first codon (ATG) of GUS is shown below (TATA box - lower case in bold; three synthetic ROS sequence - bold caps; a transcription start site - ACA, bold italics; BamHI site - GGA TCC; the first codon of GUS, ATG -italics, are also indicated):

5'-GAT ATC TCC ACT GAC GTA AGG GAT GAC GCA CAA TCC CAC TAT CCT  
TCG CAA GAC CCT TCC TCT **ata taA** TAT ATT TCA ATT TTA TTG TAA TAT  
AAT ACG GGG GAC TCT AGA GGA TCC TAT ATT TCA ATT TTA TTG TAA  
TAT AGC TAT ATT TCA ATT TTA TTG TAA TAT AAT CGA TTT CGA ACC  
CGG GGT ACC GAA TTC CTC GAG TCT AGA GGA TCC CCG GGT GGT CAG  
TCC CTT *ATG*-3' (SEQ ID NO:25)

As a control, p75-101, comprising an actin2 promoter (without any operator sequence) fused to GUS (Figure 5(A)), is also prepared.

5 The various constructs are introduced into *Arabidopsis*, as described above, and transgenic plants are generated. Transformed plants are verified using PCR or Southern analysis. Figure 6(A) show Southern analysis of transgenic plants comprising a first genetic construct, for example, p74-309 (35S-operator sequence-GUS, Figure 3(C)).

#### 10 GUS expression assays on reporter transgenic lines

In order to assess the activity of the modified regulatory regions, the level of expression of the GUS gene is assayed. Leaf tissues (approximately 10 mg) from putative positive transformants are placed into a microtitre plate containing 100 ul of GUS staining buffer (100mM KPO<sub>4</sub>, 1mM EDTA, 0.5 mM K-ferricyanide, 0.5 mM K-ferrocyanide, 0.1% Triton X-100, 1 mM 5-bromo-4-chloro-3-indolyl glucuronide), and vacuum-infiltrated for one hour. The plate is covered and incubated at 37°C overnight. Tissues are destained when necessary using 95% ethanol and color reaction is evaluated either visually or with a microscope.

20 For the modified 35S promoter, 45 lines had high GUS expression levels. These include 15 lines containing the ROS operator upstream of the TATA box, 24 lines containing the ROS operator downstream of the TATA box and six lines containing the ROS operator upstream and downstream of the TATA box. Using the actin2 promoter, 8 lines containing the ROS operator displayed high levels of GUS activity. An example  
25 of GUS expression in a plant transformed with p74-501 (actin-ROS operator sequence:GUS), is shown in Figure 8.

Single copy transformants expressing various levels of GUS activity are used for crossing with repressor lines prepared in Example 2, as outlined in Example 4.

**Example 4: Crossing of transgenic lines containing ROS repressor constructs with transgenic lines containing GUS reporter constructs.**

Transgenic *Arabidopsis* lines containing repressor constructs (second genetic constructs) are crossed with lines containing appropriate reporter (GUS) constructs (first genetic constructs). To perform the crossing, open flowers are removed from plants of the reporter lines. Fully formed buds of plants of the repressor lines are gently opened and emasculated by removing all stamens. The stigmas are then pollinated with pollen from plants of the repressor lines and pollinated buds are tagged and bagged. Once siliques formed, the bags are removed, and mature seeds are collected. Plants generated from these seeds are then used to determine the level of reporter gene (GUS) repression by GUS staining. Levels of GUS expression in the hybrid lines are compared to those of the original reporter lines. Plants showing a decrease in GUS expression levels are further characterized using PCR, Southern and Northern analysis.

Results of a cross between a transgenic line expressing synthetic ROS (p74-101 - Figure 2D) and GUS (p74-118 (Figure 5C) are presented in Figure 9.

GUS activity (Figure 9A) is only observed in plants expressing GUS (termed GUS parent in Figure 9, expressing p74-118). The plant expressing ROS (ROS parent, expressing p74-101) exhibited no GUS expression. This result is as expected, since this plant is not transformed with the GUS construct. Of interest, however, is that the plant produced as a result of a cross between the GUS and ROS parents did not exhibit GUS activity.

Northern analysis (Figure 9B) demonstrates that GUS expression is consistent with the GUS assay (Figure 9A), in that only the GUS parent expressed GUS RNA, while no GUS expression was observed in the ROS parent or the progeny arising from a cross between the ROS and GUS parents. Similarly, as expected, no ROS expression was detected in the GUS parent. ROS expression was observed in the ROS parent and in the cross between the ROS and GUS parents.

Southern analysis of the progeny of the cross between the GUS and ROS parents demonstrates that the cross comprised genes encoding both GUS and ROS (Figure 9C).

5 These data demonstrate ROS repression of a gene of interest. The progeny of the cross between the ROS and GUS parent lines, comprising both the GUS and ROS gene, expresses the ROS repressor, which binds the operator sequence thereby inhibiting the expression of the gene of interest, in this case GUS. Inhibition of GUS expression was observed at the RNA and protein levels, and no enzyme activity was present in the progeny plants.

10 These data demonstrate that expression of a gene of interest can be controlled using the repressor mediated system as described herein.

All citations are herein incorporated by reference.

15 The present invention has been described with regard to preferred embodiments. However, it will be obvious to persons skilled in the art that a number of variations and modifications can be made without departing from the scope of the invention as described herein.